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TRADITIONAL CHINESE MEDICINE TONGXINLUO PROTECTS MICROVASCULAR FROM ARTERIOLE SPASM INDUCED BY NOREPINEPHRINE IN RATS MESENTERY

Jun-Xiu Zhang[1], Shao-Dan Li[1], Yi Liu[1], Ming-Hui Yang[1]

[1]TCM Department,Chinese PLA General Hospital,Beijing 100853,China, Beijing, Haidian District, Fuxing Road, NO.28, 100853
Correspondence to: Ming-hui Yang, Department of traditional Chinese medicine, the Chinese PLA General Hospital, Beijing 100853, China. E-mail: ymh9651@sina.com.

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Abstract

Background: Arteriole spasm (AS) served as a common pathogenesis of many diseases. Tongxinluo (TXL), a traditional Chinese formula, has been proved to benefit patients with diseases associated with AS. However, the effect of TXL on AS has not been elucidated, especially on the hemorheology of microcirculation. The aim of this study was to assess the protective function of TXL against AS on microcirculation in rats' mesentery.

Materials and Methods: Arteriole spasm model was established by local administration of norepinephrine in rats' mesentery. Rats were randomized divided into five groups: sham group (local administration of normal saline), arteriole spasm group, TXL0.4+AS group, TXL0.8+AS group, TXL1.6+AS group. Drugs were administered orally daily for 7 days before modeling. Microcirculation was observed using an upright microscope and recorded to study the hemorheology changes, including spasm time, diameter changes, and arteriole blood flow. Mast cell degranulation was identified by vital staining with topical application of 0.1% toluidine blue. The serum samples were collected for detect of NO and ET-1.

Results: Pre-administration of TXL markedly decreased arteriole spasm time, and indicated a dose-dependent effect. Pre-administration of TXL also indicates dose-dependent effect to maintain arteriole diameter in the presence of AS. Pretreatment with TXL significantly inhibited mast cell degranulation induced by AS, TXL also exert great beneficial protection on arteriole blood flow against AS. The serum level of NO and ET-1 had no obvious changes among each groups.

Conclusion: AS caused microvascular dysfunction in rats' mesentery. TXL had a beneficial effect against AS in the perspective of protecting microvascular function, indicated that TXL fight against AS through regulation on microcirculation.

Key words: Arteriole spasm; Tongxinluo; Rat mesentery; Norepinephrine; Theory of TCM

Abbreviations: Tongxinluo (TXL); Arteriole spasm (AS); Traditional Chinese Medicine (TCM); Coronary artery spasm (CAS); Percutaneous coronary intervention (PCI).

Introduction

A sound microcirculation is pivotal for the physiology of the circulatory system since many essential processes take place at this segment, including delivery of nutrients, removal of waste products, and maintenance of fluid balance between blood and the

interstitium. Disorders in microcirculation result in a wide range of human or animal diseases.

Arteriole spasm causes arteriole lumen stenosis and arteriole occlusion, leading to local hemorheology disturbance and local microcirculation dysfunction. Arteriole spasms are clinically well recognized for their pathophysiological consequences in cardiology as well as in other end-organ systems, such as cardiac diseases, cerebral diseases and hypertension (Prinzmetal et al., 1959; Ishiyama et al., 2010; Grasso et al., 2004; Lin et al., 1998). Coronary artery spasm, leads to sudden cardiac arrest (Miller et al., 1982; Meisel et al., 2002; Myerburg et al., 1992; MacAlpin et al., 1993) and syncope (MacAlpin et al., 1993; Igarashi et al., 1994) subsequent to lethal arrhythmias, i.e., ventricular fibrillation, ventricular tachycardia, and atrioventricular block. Besides, arteriole spasm has been postulated as a common precipitant of acute myocardial infarction in patients with atherosclerosis (Scanlon et al., 1999). Convincing evidence suggest that intense microvascular spasm plays a key role in most cases of angiographic no-reflow, which cause Post-PCI complications (Saloum et al., 2005; Barrabes et al., 2005). Cerebral vasospasm is well known and has been investigated extensively (Dreier et al., 2002; Grasso, 2004). Cerebral arteriole spasm is involved in the pathological process of ischemia and ischemia–reperfusion period, including ischemia after hemorrhage (Ishiyama et al., 2010; Sun et al., 2009). Constant blood flow in the organs is maintained within a range of luminal pressure. When the luminal pressure increases beyond an upper limit, a breakthrough of severe vasoconstriction will occur (Strandgaard et al., 1973; Mackenzie et al., 1976; Gifford, 1991), the basic physiological process of hypertension is constriction of small arteries and arterioles (Lin et al., 1998). Leishman (Leishman, 1957), in his classic clinical studies, stated that the degree of spasm in the retinal arterioles in hypertension depends upon the height of the BP and the degree of 'involutionary sclerosis'.

It is widely accepted that norepinephrine is the neurotransmitter for sympathetic adrenergic nerves and activates α1- and α2-adrenoceptors. In the male rat mesenteric artery, but not vein, norepinephrine has been shown to cause vasoconstriction via activation through mainly postsynaptic α1-adrenoceptors (Pérez-Rivera et al., 2007). Norepinephrine leads to stimulation of vascular smooth muscle cells through the elevation of Ca^{2+} levels (Berridge, 2008). Endothelial and endothelium-derived NO in rats resistance mesenteric arteries play an important role in resisting the vasoconstriction to norepinephrine, which suggest an disordered endothelial function in the presence of norepinephrine (Xavier et al., 2011; Miyagawa et al., 2007). The mechanism of norepinephrine induced-constriction effect in rats mesenteric artery, agreed with present mechanism of CAS (Lanza et al., 2011). The use of mesenteric artery could present straightforward results with respect to artery spasm. We established mesentery microvascular spasm rats' model based on topical administration of norepinephrine (Takakura et al., 1995), and got visual parameters of artery spasm. Recent study has demonstrated Tongxinluo has an inhibitory effect against vasospasm (Guan et al., 2008). Here we present some microcirculation observation data of TXL's protective effect in the rats' mesentery arteriole spasm model.

Materials and Methods

Animals and Regents

Male Sprague-Dawley rats, weighing 180-200 g, were purchased from the Animal Center of The general hospital of Chinese People's Liberation Army (PLAGH), (Beijing, certificate no. SCXK 2012-0001). All animals were handled according to the guidelines of the PLAGH Animal Research Committee, and the surgical procedures and experimental protocol were approved by PLAGH Animal Ethics Committee.

Pentobarbital sodium was purchased from Sinopharm Chemical Reagent Co., Ltd (Batch No. 20141206). Toluidine blue was purchased from Beijing Solarbio Co., Ltd (Batch No.20140915). Norepinephrine bitartrate injection (1ml, 2mg) was purchased from Tianjinjinyao pharmacy Co., Ltd (Batch No. 1503121). Tongxinluo superfine power (TXL) was purchased from Shijiazhuang Yiling pharmacy Co., Ltd (Batch No. 20150121).

Preparation of Rats for Arteriole Spasm Model

Rats were anesthetized with 0.3% pentobarbital sodium (1ml/100g) by intramuscular injection. The abdomen was opened via a

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midline incision 20–30 mm long. The ileocecal portion of the mesentery 20 cm caudal was gently exteriorized and mounted on a custom-designed transparent plastic stage. The mesentery was kept warm and moist by continuous super-fusion with saline solution at 37°C. Microcirculatory hemodynamics in the mesentery was observed using an upright microscope (BH-2, OLYMPUS, JAPAN). The mesentery was trans-illuminated with a 12-V 100-W direct light source. The images were transmitted onto a color monitor by a video camera (Color video camera, JVC, JAPAN) mounted on the microscope and recorded with a videocassette recorder. A single un-branched arteriole with diameter between 30 and 80 um and length longer than 200 um was selected for study.

After 10 min of basal observation of the hemodynamics in the rat mesenteric microvasculature, the arteriole spasm was accomplished by dripping 50 ul (1ug/ml norepinephrine, 37℃ saline solution) on the surface of selected arteriole using a micropipette - refers to the previous research (Takakura et al., 1995). Video observation continues during the dripping, in order to record the microvascular hemodynamics.

Experimental Protocol

In the Sham and AS group, saline solution was given through intragastric at the volume of 4 ml/kg/d for 7 days. In the TXL+AS groups, TXL superfine powder dissolved in saline solution was given through intragastric at the volume of 4 ml/kg/d, with the final TXL superfine power concentration of 0.4, 0.8, and 1.6 g/kg/d for 7 days' administration, which were 1.0-, 2.0-, and 4.0-fold higher than that administered to humans in the clinic, respectively, for the three TXL pretreatment groups. The implementation of arteriole spasm was performed 2 hours right after last intra-gastric administration. A total of 50 animals were included and randomly distributed into the Sham, AS, TXL (0.4 g/kg) + AS, TXL (0.8 g/kg) + AS, and TXL (1.6 g/kg) + AS groups, with 10 animals in each group.

Measurement of Arteriole Parameters

The images of arterioles were acquired through a CVC camera system (InterVideo WinDVR). The time of arteriole spasm was calculated offline during play back of video clips, at the beginning, peak, and end of spasm. The arteriole diameter were determined using Image-Pro Plus 6.0 software (Media Cybernetic) before AS (baseline), and at the peak of spasm. The blood flow velocity generally divided into two kinds, no blood flow and remains blood flow, at the peak of arteriole spasm. The ratio of the number of no blood flow arterioles to the total number of arterioles was calculated and statistically analyzed.

Mast Cells Degranulation

Mast cells were identified by vital staining with topical application of 0.1% toluidine blue to the mesentery right after the end of spasm. The numbers of both nondegranulated and degranulated mast cells were scored from the CVC video images, and the ratio of the number of degranulated mast cells to the total number of mast cells evaluated was calculated and presented as the degranulated mast cell ratio (Han et al., 2001).

Determination of NO and ET-1

Two ml of vein blood was taken after the evaluation of mast cell degranulation in rats aorta abdominalis and centrifuged at 3000 r/min and 4℃ for 10min immediately. 0.5 ml plasma was collected and stored at -20℃ for NO determination. 2 ml of vein blood was taken and put it in a tube containing 15μl 7.5% EDTA-Na_2 and 20μl trasylol. They were mixed and centrifuged at 3000 r/min and 4℃ for 10min. Then the plasma was separated and stored at -20℃ for ET-1 determination. Plasma NO was measured by nitrate reductase. Plasma ET-1 was measured by radioimmunoassay. Steps were following the prescriptions of the kits (Beijing Puerweiye Bio-Technology Co.,Ltd).

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Statistical Analysis

Differences among groups were analyzed by one-way ANOVA, LSD method was used for multiple comparison. Qualitative data were analyzed using Fisher's exact test. A P value of less than 0.05 was considered statistically significant. All analyses were performed using the SPSS software (Version 13.0, SPSS Inc., USA).

Results

Changes in Arteriole Spasm Time

Significant alteration was observed in arteriole spasm time in AS group during the observation, and the situation changed differently in the TXL pretreated groups. The time of spasm from beginning to peak, there exist no difference in the time of spasm from beginning to peak in AS group and 3 TXL pretreated groups (see Table 1). The duration of spasm peak, spasm time was shortened to a large extent in the TXL1.6 + AS group (18.98 ± 6.16s), compared with AS group (52.09 ± 11.87s), TXL0.4 and TXL0.8 + AS group stayed the same as AS group. Recovering time from spasm peak, the time from spasm peak to complete relaxation; AS group took a long time recovering from the spasm peak (159.34 ± 43.99s), TXL0.4, TXL0.8 and TXL1.6 + AS group have reduced recovering time 107.57 ± 43.13s, 99.63 ± 25.37s and 50.58 ± 10.42s respectively, TXL1.6 + AS group showed a strongest protective effect against spasm in 3 TXL administration groups. Total spasm time, from beginning to the end. The entire process of spasm took 234.82 ± 57.26s in AS group, TXL0.4, TXL0.8 and TXL1.6 + AS group have shortened the process at 182.19 ± 38.93s, 178.78 ± 30.80s, and 89.81 ± 18.41s respectively, TXL1.6 + AS group has the shortest time against other groups. During a 10 min observation, sham group did not show any appearance of spasm (data not show).

Table 1: Arteriole spasm time

Treatment	Spasm time from beginning to peak	The duration of spasm peak	Recovering time from spasm peak	Total time of spasm
AS	20.38 ± 6.0	52.09 ± 11.87	159.34 ± 43.99	234.82 ± 57.26
TXL0.4 + AS	20.67 ± 3.63	53.96 ± 8.24	107.57 ± 43.13*#	182.19 ± 38.93*#
TXL0.8 + AS	22.06 ± 6.35	55.09 ± 10.70	99.63 ± 25.37*#	178.78 ± 30.80*#
TXL1.6 + AS	20.34 ± 9.15	18.98 ± 6.16*	50.58 ± 10.42*	89.81 ± 18.41*

Table 1: Effect of TXL0.4, TXL0.8 and TXL1.6 on arteriole spasm induced by norepinephrine in rat mesentery: A quantitative evaluation of spasm time in AS group, TXL 0.4 + AS group, TXL 0.8 + AS group, TXL 1.6 + AS group. Data are expressed as means ± SD from 10 rats. *P < 0.01 relative to AS group; #P < 0.01 relative to TXL1.6 + AS group.

Changes in Arteriole Diameter

Rats in AS group has sharply reduced arteriole diameter responded to local administration of norepinephrine, decreased 86.36 ± 6.1% compared to the baseline condition. Pretreatment with TXL0.4, TXL0.8 and TXL1.6 attenuated AS-induced arteriole diameter shrink, which showed a reduced diameter ratio of 61.93 ± 7.88%, 54.40 ± 3.72% and 44.02 ± 3.68% compare to baseline condition, respectively (Fig 1. and 2.). During a 10 min observation, sham group did not show any appearance of spasm.

Mast Cell Degranulation

Mast cell degranulation was evaluated in perivascular interstitium for different groups. Topical administration of norepinephrine evoked an apparent increase in mast cell degranulation. Quantitative analysis revealed that the norepinephrine evoked enhancement in mast cell degranulation was significantly attenuated by TXL pretreatment, and it appears to be a dose-effect

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relationship. TXL 1.6 + AS group generally eliminate the mast cell degranulation evoked by topical administration of norepinephrine, compared to sham group.

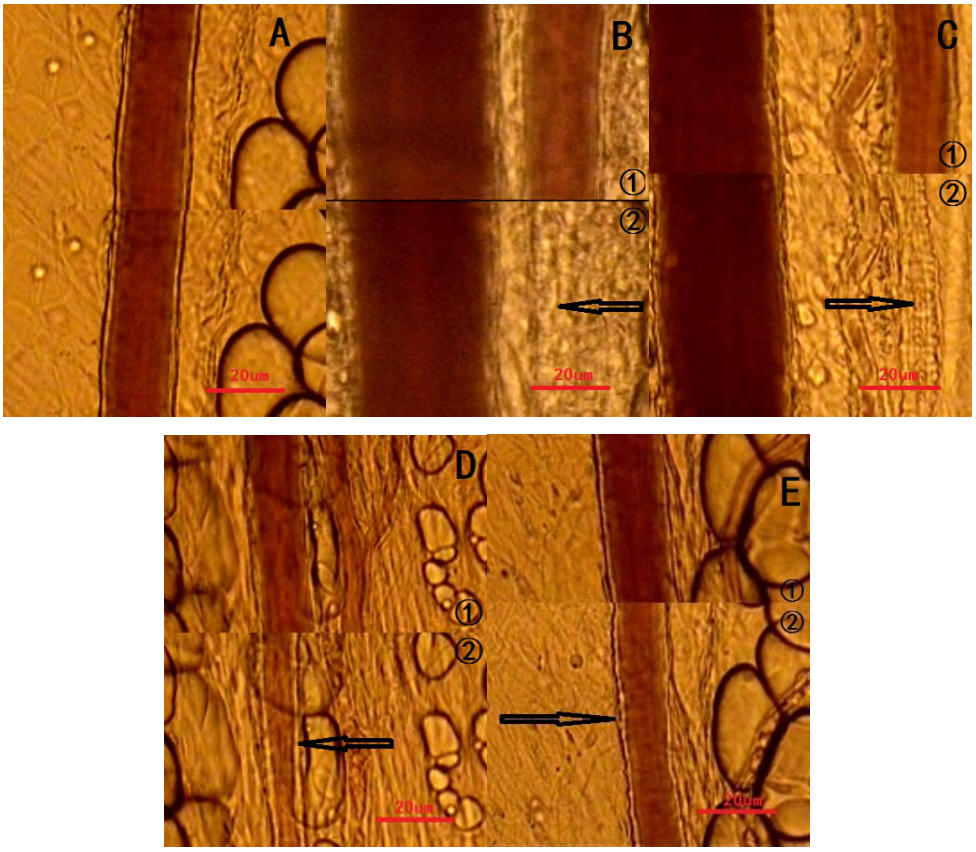

Figure 1: Representative images illustrating the effect of pretreatment with TXL on arteriole spasm induced by norepinephrine in rat mesentery. Top: rat mesentery images without administration of norepinephrine (A), Arteriole Spasm group (B), TXL0.4+AS group(C), TXL0.8+AS group (D), TXL1.6+AS group (E).① indicates baseline before administration of norepinephrine. ②: indicates arteriole diameter at the peak of spasm (scale bar = 20 um).

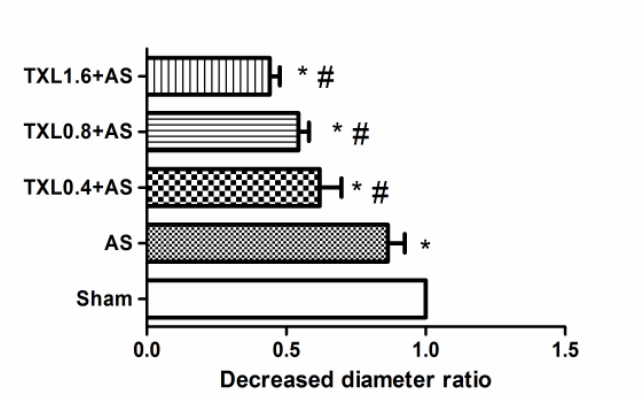

Figure 2: Effect of TXL0.4, TXL0.8 and TXL1.6 on arteriole spasm induced by norepinephrine in rat mesentery; A quantitative evaluation of decreased diameter ratio in AS group, TXL 0.4 + AS group, TXL 0.8 + AS group, TXL 1.6 + AS group. Data are expressed as means ± SD from 10 rats. * $P < 0.01$ vs. Sham group; # $P < 0.01$ vs. AS group.

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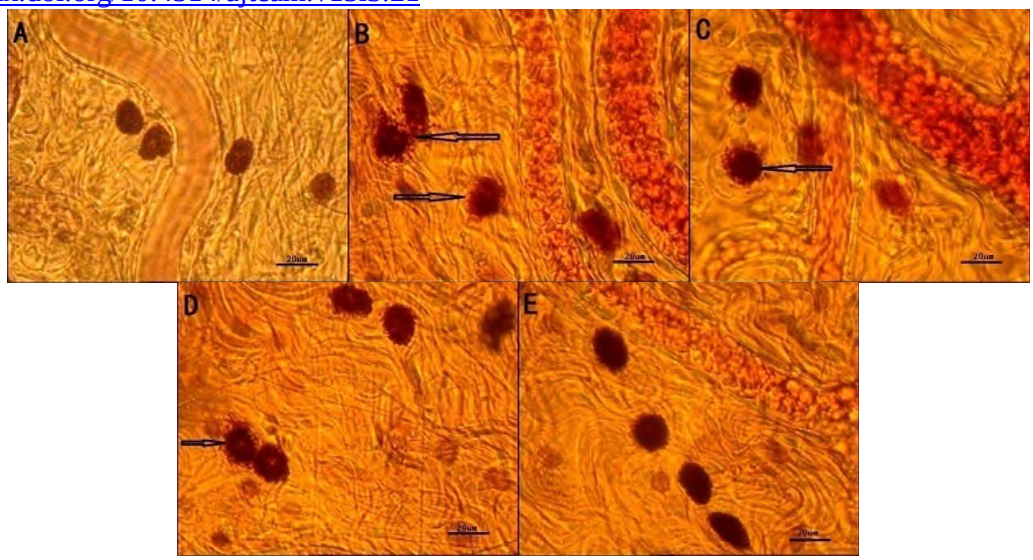

Figure 3: Representative images illustrating the effect of pretreatment with TXL on mast cell degranulation (arrow) induced by norepinephrine in rat mesentery. A: Sham group, B: Arteriole Spasm group, C: TXL0.4+AS group, D: TXL0.8+AS group, E: TXL1.6+AS group (scale bar = 20 um).

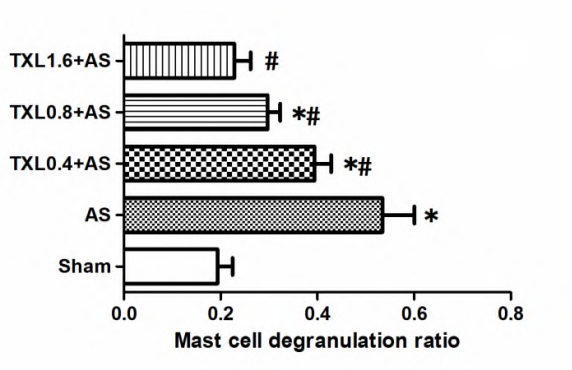

Figure 4: Effect of TXL0.4, TXL0.8 and TXL1.6 on mast cell degranulation induced by norepinephrine in rat mesentery. A quantitative evaluation of decreased diameter ratio in Sham group, AS group, TXL 0.4 + AS group, TXL 0.8 + AS group, TXL 1.6 + AS group. Data are expressed as means ± SD from 10 rats. * $P < 0.01$ vs. Sham group; # $P < 0.01$ vs. AS group.

Changes in Blood Flow Velocity

Blood flow velocity was evaluated at the peak of spasm in each group. Topical administration of norepinephrine significantly disturbed blood flow velocity. All arterioles of rats in AS group stop flowing at the peak of spasm (Fig.5. B). Pretreated with TXL largely attenuated the blocking effect of blood flow induced by norepinephrine, and it presented a dose-dependent relationship (Fig.5. A and C). All arterioles of rats in TXL 1.6 + AS group remain blood flow, presenting a strong protective effect against arteriole spasm induce by topically dripping norepinephrine.

NO, ET-1 Serum Levels

The serum level of NO in the AS group, TXL 0.4 + AS group, TXL 0.8 + AS group and TXL 1.6 + AS group didn't show any difference versus the Sham group ($P > 0.05$). Similarly, the serum level of ET-1 in the AS group, TXL 0.4 + AS group, TXL 0.8 + AS group, TXL 1.6 + AS group didn't show any difference versus the Sham group ($P > 0.05$).

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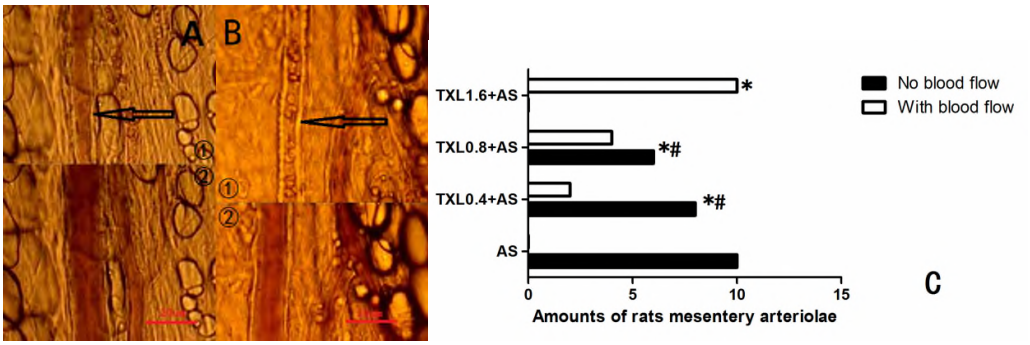

Figure 5: Effect of TXL0.4, TXL0.8 and TXL1.6 on blocked blood flow induced by norepinephrine in rat mesentery. A and B, ①: representative image of arteriole blood flow (arrow) at the peak of AS, ②: image before AS of corresponding arteriole (B①: no blood flow; A①: remains blood flow). C: black pillar indicates no blood flow in arteriole; white pillar indicates arterioles remain blood flow. A quantitative evaluation of the ratio of the number of no blood flow arterioles to the total number of arterioles in AS group, TXL 0.4 + AS group, TXL 0.8 + AS group, TXL 1.6 + AS group. Data are expressed as number of arterioles with or without blood flow from 10 rats in each group. *P < 0.01 vs. AS group; #P < 0.01 vs. TXL 1.6 + AS group (scale bar = 20 um).

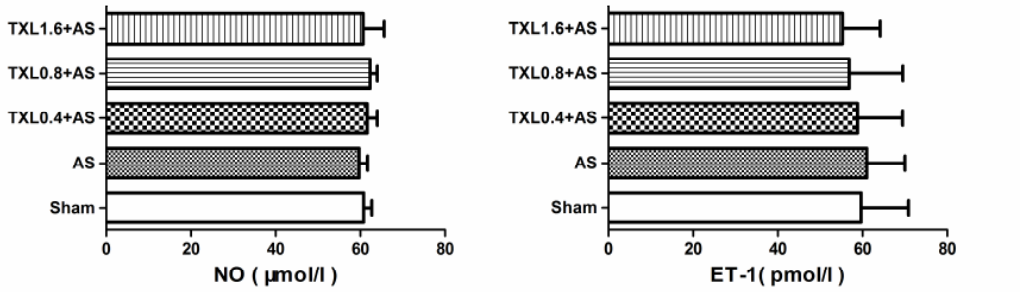

Figure 6: Effect of TXL0.4, TXL0.8 and TXL1.6 on NO, ET-1 serum levels. A quantitative evaluation of concentration in Sham group, AS group, TXL 0.4 + AS group, TXL 0.8 + AS group, TXL 1.6 + AS group. Data are expressed as means ± SD from 10 rats.

Discussion

The present study found that arteriole spasm caused target arteriole stenosis and occlusion, similar to previous study (Takakura et al., 1995), together with phenomenon of failed blood flow in peripheral venules and secondary arterioles that lead to regional ischemia and hypoxia. Arteriole spasm induced by locally administration of norepinephrine cause a local microcirculation dysfunction in rats' mesentery within about 0.8cm diameter. We generally divided arteriole spasm into 3 stages: Stage 1, spasm from beginning to the peak; Stage 2, the duration of spasm peak; Stage 3, recovery from spasm peak to normal. The time of 3 stages were used to evaluate the severity of arteriole spasm. TXL pretreatment didn't shorten Stage 1, while shortened the time of Stage 2 and 3, which may possibly indicate attenuation of spasm activated vasoconstriction reaction or augment of spasm induced vasodilatation response due to TXL (Xie et al., 2010; Guan et al., 2015). TXL's protective effects on arteriole diameter and arteriole blood flow were associated with the effect of shortened arteriole spasm process. TXL1.6 + AS group admitted the shortest arteriole spasm time and maintained largest arteriole diameter with blood flow, which may account for a protective effect against microcirculation dysfunction induced by arteriole spasm in terms of visualization outcomes in vivo. Serum NO and ET-1 concentration in the AS and TXL pretreated groups stayed the same as Sham group. Since NO is a well-known vasodilator and ET-1 is a well-documented vasoconstrictor, serum results is contradictory to the results in vivo. Considering that the arteriole spasm is

induced in the transparent thin film tissue of rats mesentery within about 0.8 cm diameter, and the target arteriole diameter is 30 ~ 80μm. The local transient arteriole spasm may not severe enough to cause changes in serum factors, that's may explain the contradiction.

Mast cells are widely distributed throughout the mammalian tissues with a preferential location in the vicinity of blood vessels (Kaliner, 1980). Haas and Bergofsky (Hass et al., 1972) found that the perivascular mast cells degranulated in response to hypoxia. And mast cell degranulation after I/R exerts insults in microcirculation (Han et al., 2001). Mast cell degranulation indirectly leads to augmented NE release (Seyedi et al., 1997; Reid et al., 2004), however, there's no obvious evidence that NE leads to mast cell degranulation. Based on this, we evaluated whether arteriole spasm could causes local hypoxia reaction and similar I/R injury in the present of mast cells. It was shown that AS group had an obvious augment of mast cell degranulation, and TXL pretreated groups attenuated the mast cell degranulation induced by arteriole spasm; besides, TXL1.6 + AS group significantly reduced mast cell degranulation. Results indicate that arteriole spasm could induce local hypoxia and similar I /R injury in the terms of mast cell degranulation. TXL pretreatment could strikingly attenuated mast cell degranulation, possibly due to the protective effect against arteriole spasm which have discussed above.

TXL is a compound prescription formulated according to the meridian theory of traditional Chinese medicine. TXL is extracted, concentrated, and freeze-dried from a group of traditional Chinese medicine. TXL had been approved for the market by the State Food and Drug Administration of China in 1996 (state medical license NO. Z20060322). The active ingredients of TXL capsule that are responsible for its effects in the study are unclearly, which may be due to the cumulative or synergistic effects of multiple compounds present in the herbal extract. For example ginseng is the major ingredient of TXL and contains a group of triterpene glycosides called ginsenosides. It has been reported that ginsenoside Rb1 attenuates ET-1-induced contractile response via inhibition of store-operated Ca(2+) entry, and it can effectively antagonize the enhanced pulmonary vasoreactivity in pulmonary hypertension (Wang et al., 2015). Ginsenoside Rb1 blocks vasculature thickening and spasm after subarachnoid hemorrhage in rats (Li et al., 2011). And that ginsenoside Rb3 exhibited similar potency in improving endothelium dependent relaxation, inhibiting endothelium dependent contractions, reducing ROS over-production and NADPH oxidase expression in renal arteries and increasing NO production and eNOS phosphorylation in human endothelial cells (Wang et al., 2014). Other study has found that paeonia has vasodilatory capacity (Ghayur et al., 2008). Besides, ginsenosides have been shown to concentration-dependently relax the prostaglandin F2a-induced contraction of isolated rabbit pulmonary arteries and the phenylephrine-induced contraction of isolated rabbit and rat aortas (Kim et al., 1994; Chen et al., 1984). TXL1.6 pretreatment exhibited greater protective effect than TXL0.4 and TXL0.8 groups is supposed to associate with increased chemical ingredients of ginsenosides.

Recent studies have demonstrated that TXL has pleiotropic effects including improvement of endothelial function, lipid lowering, anti-oxidation, vasodilatation, anti-thrombosis, anti-inflammation, anti-apoptosis, and enhancement of angiogenesis (Li et al., 2006; Liu et al., 1997; Wu et al., 2007; Zhang et al., 2008), which may be due to the cumulative or synergistic effects of multiple compounds present in the extract. Multiple function of TXL in treatment of cardiovascular and cerebrovascular disease makes it a representative formula of TCM. Diagnosis and treatment theory of TCM located in "holism" and "treatment based on syndrome differentiation ", in which "Zheng" is an important part.

"Zheng" is a concept in TCM describes the same pathological condition in diseases, and a series of symptoms reflect "Zheng". Treatment focus on the same "Zheng" leads to application to different diseases. Arteriole spasm is a pathological process in many diseases, and a series of mechanism lead to arteriole spasm. Arteriole spasm meets the standard of "Zheng". Our study focus on arteriole spasm indicates same pathological process in cardiovascular diseases as well as diseases in other end-organ systems. We proved TXL could attenuate disturbance vascular function induced by AS, and exerted an anti-hypoxia effect in vivo. In a conclusion, TXL has a beneficial effect against AS, indicates that TXL fight against AS through regulation on microcirculation.

Study Limitations

Our study had limited area of arteriole spasm and hence to show the protective mechanism of TXL we need experiments with

tissues or organs to present arteriole spasm. We did not have an active constituent analysis of TXL, and study focus on monomer of one herb like ginsenosides in ginseng will begins the work.

Conflict of Interest: The authors declare that they have no conflict of interests.

Authors' Contribution

Jun-Xiu Zhang and Yi Liu conducted the experiment. Jun-Xiu Zhang provided the reagents and materials. Shao-Dan Li and Ming-Hui Yang designed the experiment and modified the paper.

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